

Germination of Spores from *Clostridium botulinum* B-aphis and Ba410†

The germination of spores from *Clostridium botulinum* B-aphis and Ba410 was examined. In a complex medium, heat activation of spores from both strains doubled the germination rates and was required for germination in the presence of 2% NaCl. In a defined medium (CTB [D. B. Rowley and F. Feeherry, J. Bacteriol. 104:1151-1157, 1970]), the parent strain B-aphis germinated at a rate of 0.77% min⁻¹ in the absence of NaCl and was not affected by 2% NaCl. A salt-tolerant derivative, strain Ba410, germinated at rates of 0.16% min⁻¹ in CTB and 0.04% min⁻¹ in CTB containing 2% NaCl. L-Alanine-triggered spores germinated faster than did L-cysteine-triggered spores from both strains. When both amino acids were present, B-aphis germinated rapidly in the absence of NaCl and had biphasic kinetics in the presence of NaCl. Strain Ba410 had biphasic kinetics in the absence of NaCl and germinated slowly with single-phase kinetics in the presence of NaCl. L-Alanine- and L-cysteine-triggered germinations were each inhibited by both D-alanine and D-cysteine, indicating a common germinant-binding site for both alanine and cysteine. Attempts to select for variants with amino acid-specific germinant-binding sites were unsuccessful. Differences in the germination kinetics of both strains could not be explained by ultrastructural differences. Transmission electron micrographs revealed striking similarities between the strains.

Complete inhibition of botulin toxinogenesis requires NaCl levels of 5% for type E strains (2, 31) and up to 8 and 10% for type A and proteolytic B strains (1, 26) under otherwise optimal conditions. Lower salt concentrations are, however, inhibitory when used with another inhibitor (10, 32). Complex interactions among pH, salt, and nitrite are responsible

for the safety of cured meats (10, 13, 27). Thus, the antibotulin activities of even small salt concentrations should be considered during the current movement to reduce dietary sodium chloride levels.

Relatively little work has been done on the influence of NaCl on clostridial spore germination (2, 4, 26). While examining the interaction of pH and salt on botulin physiology (22, 24), we isolated and characterized a salt-tolerant variant of *Clostridium botulinum* B-aphis. This variant, strain Ba410, differs from the parent strain in salt sensitivity,

TABLE 1. Trigger specificity of six strains of *C. botulinum* spores after five rounds of selection against the ability to germinate in a given amino acid

Parent strain	Germination no. 5 strain no.	Germinant selected against	Extent ^a of amino acid-triggered germinations in medium with (condition) ^b :									
			None (1)	D-Ala (2)	D-Cys (3)	L-Cys + D-Cys (4)	L-Ala + D-Ala (5)	L-Cys + D-Ala (6)	L-Ala + D-Cys (7)	L-Ala (8)	L-Cys (9)	L-Ala + L-Cys (10)
B-aphis	115	Ala	0	3	0	0	0	1	0	42	4	24
	125	Cys	0	0	0	0	0	0	0	54	13	40
	135	Ala + Cys	0	0	1	4	1	5	4	67	17	57
Ba410	215	Ala	0	1	2	1	0	0	0	40	0	19
	225	Cys	0	1	1	1	3	6	6	91	29	85
	235	Ala + Cys	0	0	0	1	4	0	7	76	16	68

^a Percent increase of phase-dark spores in medium with germinant (s) minus percent increase of phase-dark spores in medium without germinant.

^b Heat-activated (80°C, 30 min) spores were incubated at 35°C in a medium containing sodium carbonate (11.9 mM), sodium thioglycolate (4.4 mM), and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (100 mM) and L-amino acids (10 mM), D-alanine (300 mM), and D-cysteine (150 mM) as indicated.

protease activity, regulation of cell lysis, and the germination rates of alanine- and cysteine-triggered spores (23). The objective of the present study was to compare the spore germination kinetics of *C. botulinum* B-aphis and Ba410 for different germinants and in the presence or absence of salt.

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TABLE 2. Influence of NaCl on germination of heat-activated *C. botulinum* B-aphis and Ba410 spores in CTB containing L-alanine and L-cysteine as germinants

Strain	Medium	Germination rate (% min ⁻¹) (+σ) ^a	% Germinated at 24 h
B-aphis	CTB	0.77 ± 0.03	99
	CTB + 2% salt	0.69 ± 0.07	88
Ba410	CTB	0.16 ± 0.03	85
	CTB + 2% salt	0.04 ± 0.01	34

^a Averages of triplicate determinations.

MATERIALS AND METHODS

Spore production. Spores were produced in a medium containing 5% Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.5% Difco Proteose Peptone (Difco Laboratories, Detroit, Mich.), 0.125% K₂HPO₄, and 0.075% NaHCO₃ (3). After 21 days at 30°C, the spores were harvested, cleaned by differential centrifugation, and suspended in sterile deionized water.

Generation of trigger-specific mutants. We attempted to isolate mutants of strains B-aphis and Ba410 that could be triggered by alanine but not cysteine, by cysteine but not alanine, or by neither amino acid. A strategy similar to that used by Rossignol and Vary (28) in making *Bacillus megaterium* mutants that would not germinate on proline was used. Spores from both strains were heat activated (80°C, 60 min) and transferred to a chemically defined germination medium (CTB; see below), which contained L-alanine, L-cysteine, or both amino acids as germinants. After 24 h, the spore suspensions were heated again for 60 min at 80°C to kill germinated spores. Surviving spores, i.e., those incapable of rapid germination on the germinant in the CTB, were inoculated into TP broth (30) to produce new spore crops that should be enriched for the inability to germinate on the selecting germinant. These spore crops received four additional cycles of heat activation, exposure to different germinants, and killing of germinated spores followed by spore production from the survivors.

Germination conditions. Germination kinetic experiments were done in the CTB of Rowley and Feeherry (29) with 4.5 mM L-alanine, 32 mM L-cysteine, or both amino acids as germinants. The concentrations of amino acids in the germinant-binding-site specificity experiments are given in Table 1. Botulinum assay medium (BAM) (21) was the complex germination medium. Spore germination at 35°C was followed, with loss of phase brightness as the criterion, and by procedures previously reported (23).

Transmission electron microscopy. Spore suspension (2 ml) was heated at 80°C for 30 min and then centrifuged in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) for 2 min. The spores were fixed in 1.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 6.6) for 1 h at ambient temperature and then were rinsed overnight at 4°C in sodium cacodylate buffer (pH 6.8) followed by two short rinses in the same buffer. In each case, the spores were centrifuged and resuspended in the next solution. The spores were postfixed in 1% osmium tetroxide in sodium cacodylate buffer for 1 h at ambient temperature, rinsed briefly in sodium cacodylate buffer, and then enrobed in agar by mixing 3 drops of warm 2% agar into the drained pellet. When hardened, the agar was cut into pieces of approxi-

mately 2 mm³, dehydrated in a graded ethanol series, and embedded in EMBed-812 epoxy resin (Electron Microcopy Sciences, Fort Washington, Pa.). Thin sections were stained with uranyl acetate and lead citrate.

RESULTS AND DISCUSSION

Experiments on spores germinated in CTB with L-alanine and L-cysteine as the germinants (Table 2) confirmed our earlier observations (23). Strain B-aphis germinated at a rate of 0.77% min⁻¹. Within 24 h, 99% of the spores germinated. Such rapid germination appears to be typical of *C. botulinum*, having been observed with cysteine-triggered spores of strain 62A (29), alanine-triggered spores of strain 12885A (5), spores of strain 62A germinated in complex medium (36), and cysteine-triggered spores of the closely related *Clostridium sporogenes* (35). The addition of 2% salt to the medium had an insignificant effect on the germination rate, but the extent of germination was slightly, but consistently, decreased. Ando (1) demonstrated that increasing salt concentrations from 0 to 8% progressively reduced the rate and extent of germination of type A spores. The relative effect was similar to that reported here; 2% salt reduced the extent of germination from 91 to 81%. Ba410 spores germinated at a slow rate, 0.16% min⁻¹, but 85% did germinate within 24 h. In CTB plus 2% salt, both the rate and extent of germination were markedly reduced. Neither quadrupling the alanine and cysteine concentrations nor doubling the bicarbonate concentration increased germination (data not shown).

Because salt sensitivity can be a manifestation of injury (14, 25), and injured spores may have additional germination requirements (5, 6), the germination of heat-activated and unheated spores was examined in BAM and BAM plus 2% NaCl (BAM/S). In BAM, unheated B-aphis spores started to germinate after a long lag period (Fig. 1). Once started, germination proceeded at a linear rate for about 80 min, but only 25% of the spores germinated. Unheated Ba410 spores started to germinate immediately upon exposure to the medium, but only 13% of the population germinated. Germination could not be followed for more than 3 h in BAM because of replicating vegetative cells. Additional germination at a slow rate must occur, since B-aphis spores exhibit maximal colony formation on BAM agar in the absence of heat activation (21). In BAM/S, germination of unheated spores from both strains was not observed. A low level of

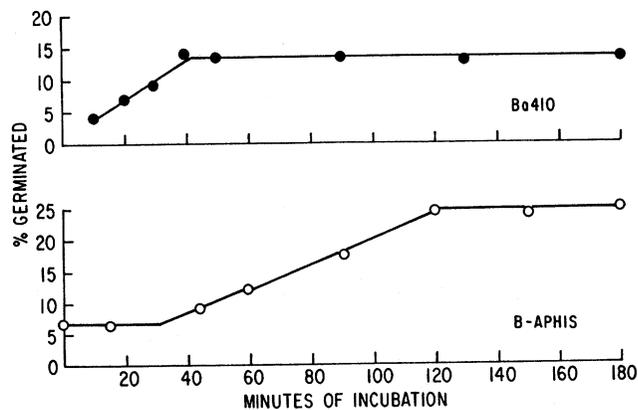


FIG. 1. Extent of germination versus time for *C. botulinum* Ba410 (●) and B-aphis (○) spores. Unheated spores were incubated with BAM at 37°C. The proportion of phase-dark (i.e., germinated) spores was determined for samples taken at the times indicated.

germination, not detected by the methods used here, may have occurred since about 10% of *C. botulinum* 62A spores germinate and form colonies on BAM/S agar (24).

Heat activation increased both the rate and extent of germination for both strains (Fig. 2 and 3). B-aphis spores germinated rapidly in BAM. In BAM/S, there was a period of rapid germination followed by a 40- to 60-min lag, and then another period of rapid germination. Although the length of the secondary lag phase varied somewhat, the biphasic curve was observed in all trials. Heat-activated Ba410 spores exhibited a biphasic germination pattern in BAM (Fig. 3). In BAM/S, the spores germinated rapidly, but plateaued when less than 40% of the population germinated. The role of heat activation in changing germination rates and the composition, morphology, permeability, and metabolic activity of spores has been reviewed (15).

The biphasicness reported here is different from that observed by Hashimoto et al. (8, 9). They measured germination by the optical density change of individual bacillus spores. The individual spore rapidly changed from phase bright to phase gray, and then after a period of phase grayness rapidly became phase dark.

Germination rates of spores under the conditions described for Fig. 1 through 3 were calculated from the linear portions of germination response curves of triplicate experiments (Table 3). In basal medium, unheated spores of both strains germinated at similar rates significantly ($P < 0.05$) slower than the first linear period of heat-activated spores. Salt inhibited the germination of unheated spores. The germination rate of heat-activated B-aphis spores in the NaCl-containing medium during the first and second linear periods was similar to that of spores in basal medium. The germination rates of heat-activated Ba410 spores were influenced by salt. When salt was absent, the germination rate during the first period was comparable to that of the heat-activated B-aphis spores but was followed by a second, slower, period of germination. The germination rates of heat-activated Ba410 spores were reduced in the presence of salt; again, the first germination period was significantly faster than the second.

Spore germination is a multistep process that consists of a

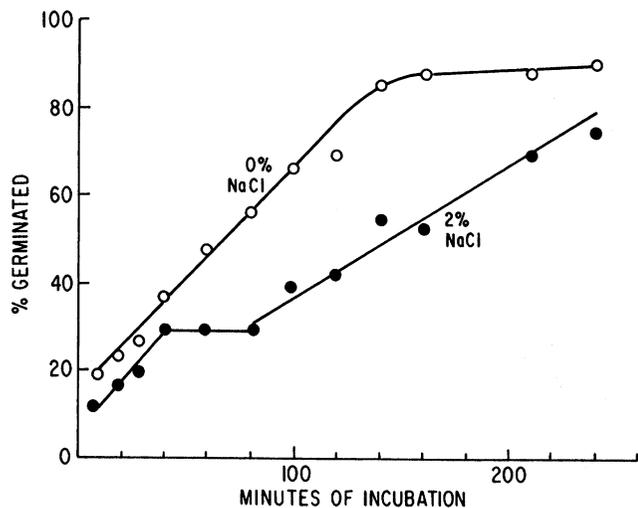


FIG. 2. Extent of germination versus time for heat-activated (80°C, 30 min) *C. botulinum* B-aphis spores in BAM with 0 or 2% NaCl.

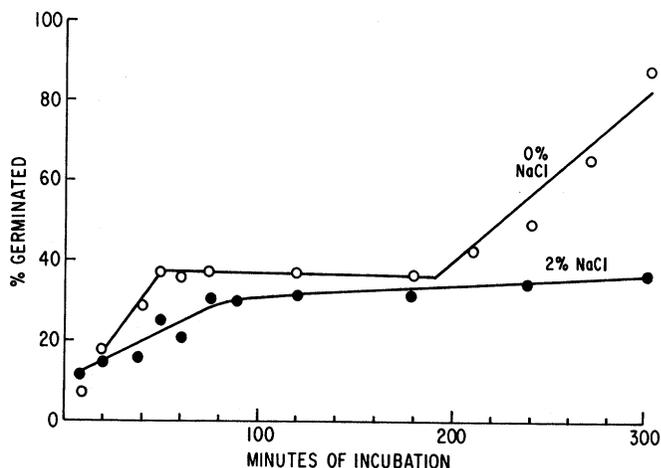


FIG. 3. Extent of germination versus time for heat-activated *C. botulinum* Ba410 spores in BAM with 0 or 2% NaCl.

trigger reaction in which a germinant binds to an active site and produces an allosteric effect (4, 32), setting off the connecting reactions that change spore properties. These changes culminate in depolymerization of the cortex (7), and spores become phase dark. To determine if the biphasic germination kinetics might be due to different trigger specificities, B-aphis and Ba410 spores were germinated in CTB with L-alanine, L-cysteine, or both amino acids as triggers. The germination of heat-activated B-aphis spores triggered by L-alanine alone or by L-alanine plus L-cysteine was rapid (Fig. 4). Within 24 h, 99% of the spores germinated. The absence of a lag period suggested that both the triggering and connecting reactions proceeded rapidly. In contrast, spores exposed to L-cysteine alone germinated more slowly and had biphasic kinetics. Within 24 h, 82% of the spores germinated. The differences between L-alanine- and L-cysteine-triggered germination kinetics suggested that these germinants trigger mechanistically dissimilar connecting reactions.

Ba410 spores responded to these germinants differently. Spores exposed to L-alanine alone gave a biphasic response (Fig. 5); 84% germinated within 24 h. Ba410 spores exposed simultaneously to L-alanine and L-cysteine germinated linearly, with 78% germinating within 24 h. When L-cysteine was the sole germinant, a portion of the population germinated with linear kinetics, but this period lasted only 120 min. Only 48% of these spores germinated within 48 h. Neither strain demonstrated appreciable autogermination

TABLE 3. Effect of heat activation on germination of *C. botulinum* B-aphis and Ba410 spores in BAM

Heat activation (80°C, 30 min)	2% salt	Germination rate (% min ⁻¹)			
		B-aphis		Ba410	
		Linear period 1	Linear period 2	Linear period 1	Linear period 2
-	-	0.20 ^{a,§§}	NO ^b	0.28 ^{§§}	NO
-	+	— ^c	NO	—	NO
+	-	0.50 [*]	NO	0.50 [*]	0.36 ^{††}
+	+	0.47 [*]	0.42 ^{*†}	0.33 ^{††}	0.09

^a Means in columns and rows with the same symbol are not statistically different at the 0.05 level in the Duncan's range test.

^b NO, Not observed.

^c —, No germination observed within 3 h.

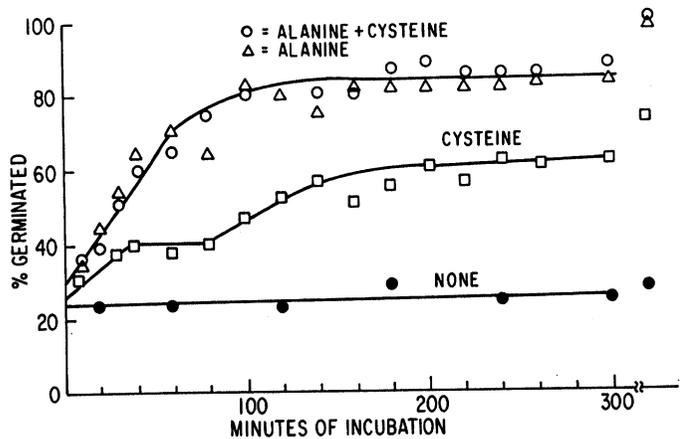


FIG. 4. Extent of germination versus time for heat-activated *C. botulinum* B-aphis spores in CTB that contained L-alanine, L-cysteine, both amino acids, or no germinant.

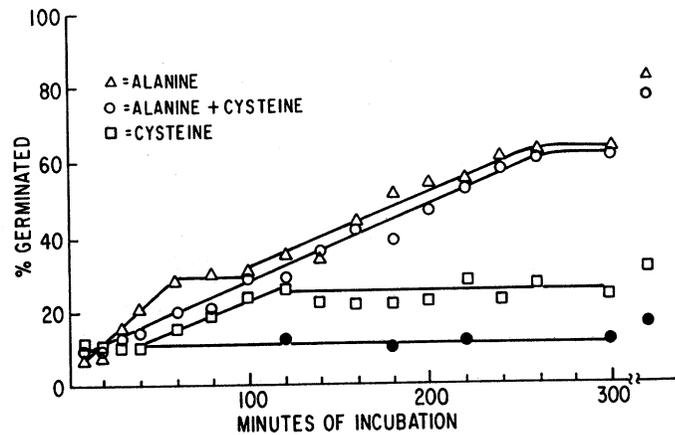


FIG. 5. Extent of germination versus time for heat-activated *C. botulinum* Ba410 spores in CTB that contained L-alanine, L-cysteine, both amino acids, or no germinant.

when incubated for 24 h in CTB containing no germinant. The germination response of Ba410 spores to L-alanine plus L-cysteine had neither the lag period characteristic of L-alanine-triggered germination nor the early plateau characteristic of L-cysteine-triggered germination. We hypothesized that biphasic germination kinetics might be due to different germinant-binding sites that are specific for either cysteine or alanine. If this was true, spores produced by successive transfer after exposure to a given germinant (i.e., alanine) and to heating would become enriched for the alternate germinant-binding site (i.e., cysteine triggering). Conversely, if both amino acids activated the same germinant-binding site, then the germination rate would be determined by the total number of germinant-binding sites, and no enrichment for germinant specificity should occur.

Five successive selection cycles were used to kill spores that germinated in response to a specific amino acid. The survivors were then used as inocula for another round of spore production. This did not result in the selection of germinant-specific spores (Table 1). All of the strains generated by the selection procedure showed germinant efficiencies similar to those of their parent strains. The greatest extent of germination occurred in the presence of L-alanine (condition 8 in Table 1) and the slowest in the presence of L-cysteine (condition 9 in Table 1); L-alanine plus L-cysteine (condition 10 in Table 1) gave an intermediate response. Quantitative differences in the extent of germination of spores derived from the same parent strain (i.e., strains 115, 125, and 135, all derived from B-aphis, and 215, 225, and 235, all derived from Ba410) could be attributed to the normal variations observed among different spore crops of the same strain. None of the strains germinated in the absence of an L-amino acid germinant (conditions 1 through 3 in Table 1). D-Cysteine inhibited both L-cysteine- and L-alanine-triggered germination (conditions 4 and 7 in Table 1). D-Alanine inhibited both L-cysteine- and L-alanine-triggered germination (conditions 6 and 5 in Table 1).

The data suggest that botulin spores have only one germinant-binding site for which alanine is a better germinant than is cysteine. The single germinant-binding-site model is supported by the observation that L-cysteine-triggered germination was inhibited by D-alanine as well as by D-cysteine and that L-alanine-triggered germination was inhibited by D-cysteine as well as by D-alanine. These results agree with reports that D-alanine inhibits L-alanine-triggered

germination of putrefactive anaerobe 3679h spores (37) and type A botulin spores (1). Tang and Frank (35) demonstrated that D-alanine inhibits cysteine-triggered germination of putrefactive anaerobe 3679h spores. A series of elegant experiments by Blocher and Busta (J. Blocher, Ph.D. thesis, University of Minnesota, St. Paul, 1984) have shown that the triggering of botulin spores rather than the subsequent connecting reactions is the site of D-alanine inhibition of L-alanine-induced germination.

Having determined that the differences in germination kinetics were not due to differences in germinant-binding-site specificity, we sought to determine whether strain B-aphis and Ba410 spores had gross ultrastructural differences. Examination of electron micrographs of over 300 cells showed that both spore crops had similar distributions of cell types (Table 4).

The ultrastructure of spores encased in exosporium (Fig. 6) was virtually identical for both strains and was typical for clostridial spores, although more oval than the botulin spores observed by Stevenson et al. (34). A fuzzy layer was attached to the outer surface of the exosporium, which had a laminar structure similar to that observed with type A botulin spores (33) and other clostridia (18, 20). The opening at the base of the exosporium is the site of emergence for clostridial cells (19), which, unlike *Bacillus* spores, germinate within the exosporium (12). The easily differentiated subcoat and overcoat were similar to those of *Clostridium perfringens* (17). There is a close association of the germination lytic system with the subcoat region (16). Although the difference was not dramatic, the subcoats of

TABLE 4. Cell types in electron micrographs of *C. botulinum* B-aphis and Ba410 21-day-old cultures

Cell type	% Distribution in strain:	
	B-aphis	Ba410
Spores in exosporium	55	52
Vegetative cells with or without spores	21	29
Empty exosporium and membrane fragments	22	18



FIG. 6. Ultrastructure of *C. botulinum* B-aphis (a) and Ba410 (b) spores, $\times 75,000$ magnification. F, Fuzzy layer; EX, exosporium; \square , spore coats that include an outer coat, a dense ribbonlike coat, and a subcoat; CX, cortex; GCW, germ cell wall, which abuts an inner forespore membrane; CR, core, which contains ribosomes and nucleus.

Ba410 spores were somewhat less dense than those of B-aphis spores.

In summary, we observed different germination patterns in spores of *C. botulinum* B-aphis and Ba410. The trigger specificities of both strains were similar; D-alanine and D-cysteine inhibited both L-alanine- and L-cysteine-triggered germination. Spores of both strains had similar ultrastructures. By the process of elimination, it appears that the connecting reactions may be the site of the difference between these two strains. Connecting reactions in clostridial spore germination would undoubtedly be a fertile field for further study.

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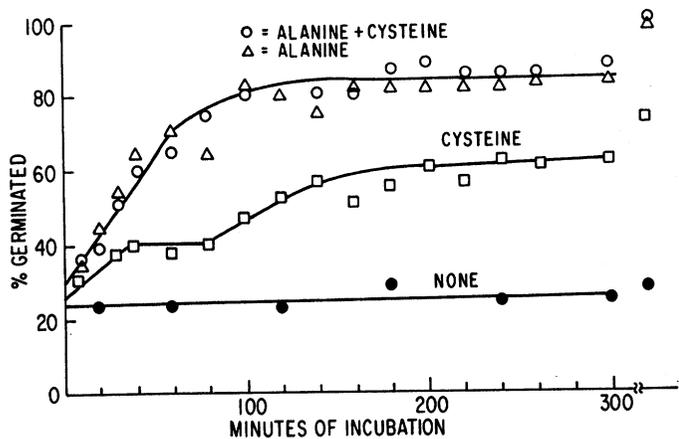


FIG. 4. Extent of germination versus time for heat-activated *C. botulinum* B-aphis spores in CTB that contained L-alanine, L-cysteine, both amino acids, or no germinant.

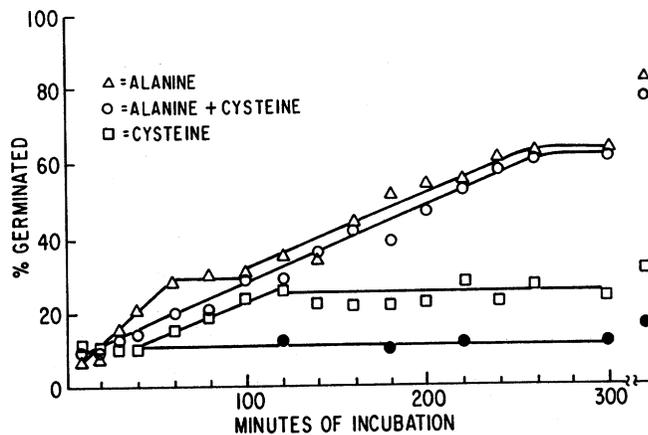


FIG. 5. Extent of germination versus time for heat-activated *C. botulinum* Ba410 spores in CTB that contained L-alanine, L-cysteine, both amino acids, or no germinant.

when incubated for 24 h in CTB containing no germinant. The germination response of Ba410 spores to L-alanine plus L-cysteine had neither the lag period characteristic of L-alanine-triggered germination nor the early plateau characteristic of L-cysteine-triggered germination. We hypothesized that biphasic germination kinetics might be due to different germinant-binding sites that are specific for either cysteine or alanine. If this was true, spores produced by successive transfer after exposure to a given germinant (i.e., alanine) and to heating would become enriched for the alternate germinant-binding site (i.e., cysteine triggering). Conversely, if both amino acids activated the same germinant-binding site, then the germination rate would be determined by the total number of germinant-binding sites, and no enrichment for germinant specificity should occur.

Five successive selection cycles were used to kill spores that germinated in response to a specific amino acid. The survivors were then used as inocula for another round of spore production. This did not result in the selection of germinant-specific spores (Table 1). All of the strains generated by the selection procedure showed germinant efficiencies similar to those of their parent strains. The greatest extent of germination occurred in the presence of L-alanine (condition 8 in Table 1) and the slowest in the presence of L-cysteine (condition 9 in Table 1); L-alanine plus L-cysteine (condition 10 in Table 1) gave an intermediate response. Quantitative differences in the extent of germination of spores derived from the same parent strain (i.e., strains 115, 125, and 135, all derived from B-aphis, and 215, 225, and 235, all derived from Ba410) could be attributed to the normal variations observed among different spore crops of the same strain. None of the strains germinated in the absence of an L-amino acid germinant (conditions 1 through 3 in Table 1). D-Cysteine inhibited both L-cysteine- and L-alanine-triggered germination (conditions 4 and 7 in Table 1). D-Alanine inhibited both L-cysteine- and L-alanine-triggered germination (conditions 6 and 5 in Table 1).

The data suggest that botulin spores have only one germinant-binding site for which alanine is a better germinant than is cysteine. The single germinant-binding-site model is supported by the observation that L-cysteine-triggered germination was inhibited by D-alanine as well as by D-cysteine and that L-alanine-triggered germination was inhibited by D-cysteine as well as by D-alanine. These results agree with reports that D-alanine inhibits L-alanine-triggered

germination of putrefactive anaerobe 3679h spores (37) and type A botulin spores (1). Tang and Frank (35) demonstrated that D-alanine inhibits cysteine-triggered germination of putrefactive anaerobe 3679h spores. A series of elegant experiments by Blocher and Busta (J. Blocher, Ph.D. thesis, University of Minnesota, St. Paul, 1984) have shown that the triggering of botulin spores rather than the subsequent connecting reactions is the site of D-alanine inhibition of L-alanine-induced germination.

Having determined that the differences in germination kinetics were not due to differences in germinant-binding-site specificity, we sought to determine whether strain B-aphis and Ba410 spores had gross ultrastructural differences. Examination of electron micrographs of over 300 cells showed that both spore crops had similar distributions of cell types (Table 4).

The ultrastructure of spores encased in exosporium (Fig 6) was virtually identical for both strains and was typical for clostridial spores, although more oval than the botulin spores observed by Stevenson et al. (34). A fuzzy layer was attached to the outer surface of the exosporium, which had a laminar structure similar to that observed with type A botulin spores (33) and other clostridia (18, 20). The opening at the base of the exosporium is the site of emergence for clostridial cells (19), which, unlike *Bacillus* spores, germinate within the exosporium (12). The easily differentiated subcoat and overcoat were similar to those of *Clostridium perfringens* (17). There is a close association of the germination lytic system with the subcoat region (16). Although the difference was not dramatic, the subcoats of

TABLE 4. Cell types in electron micrographs of *C. botulinum* B-aphis and Ba410 21-day-old cultures

Cell type	% Distribution in strain:	
	B-aphis	Ba410
Spores in exosporium	55	52
Vegetative cells with or without spores	21	29
Empty exosporium and membrane fragments	22	18

